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THE EFFECT OF SAQUINAVIR ON THE RATE OF METABOLISM OF MIDAZOLAM

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## ABSTRACT

Midazolam (MDZ) is used extensively for sedation by clinicians including anesthesia providers. There have been reports that its effects have been enhanced in the presence of other drugs with negative patient outcomes. The P450 mixed function oxidase system contains an enzyme subfamily known as CYP3A. These enzymes have been identified as primarily responsible for the metabolism of MDZ. Protease inhibitors such as saquinavir may inhibit some P450 isoforms. The metabolism of MDZ in the presence of protease inhibitors is presently unclear. This study examines the effect of saquinavir on the rate of metabolism of MDZ. Human liver microsomes were incubated with MDZ with and without saquinavir. The ratio between MDZ's major metabolite α-hydroxy MDZ and the internal standard lorazepam were obtained using high performance liquid chromotography (HPLC). These ratios were then compared to those results in the presence of saquinavir. The incubation concentrations of MDZ were similar to therapeutic concentrations of (0.5μM, 1μM, 3μM, 6μM, 12μM) and saquinavir (0.0μM, 0.3μM, 1.0μM, 3.0μM and 10.0μM). MDZ was inhibited 56.73% ±5.63. The Ki was determined to be 3.4 μM. The interaction exhibited properties of mixed inhibition. These results showed that the rate of metabolism of MDZ was statistically significantly decreases in the presence of saquinavir ($p<.05$).



Key Words: **protease inhibitor saquinavir midazolam metabolism**

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## ABSTRACT

Midazolam (MDZ) is used extensively for sedation by clinicians including anesthesia providers. There have been reports that its effects have been enhanced in the presence of other drugs with negative patient outcomes. The P450 mixed function oxidase system contains an enzyme subfamily known as CYP3A. These enzymes have been identified as primarily responsible for the metabolism of MDZ. Protease inhibitors such as saquinavir may inhibit some P450 isoforms. The metabolism of MDZ in the presence of protease inhibitors is presently unclear. This study examines the effect of saquinavir on the rate of metabolism of MDZ. Human liver microsomes were incubated with MDZ with and without saquinavir. The ratio between MDZ's major metabolite α-hydroxy MDZ and the internal standard lorazepam were obtained using high performance liquid chromotography (HPLC). These ratios were then compared to those results in the presence of saquinavir. The incubation concentrations of MDZ were similar to therapeutic concentrations of (0.5μM, 1μM, 3μM, 6μM, 12μM) and saquinavir (0.0μM, 0.3μM, 1.0μM, 3.0μM and 10.0μM). MDZ was inhibited 56.73% ±5.63. The Ki was determined to be 3.4 μM. The interaction exhibited properties of mixed inhibition. These results showed that the rate of metabolism of MDZ was statistically significantly decreases in the presence of saquinavir ($p<.05$).



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# THE EFFECT OF SAQUINAVIR ON THE RATE OF METABOLISM OF MIDAZOLAM

Brian G. Todd, Capt, BSN

THESIS
Presented to the Graduate School of Nursing Faculty of

The Uniformed Services University of the Health

Sciences in Partial Fulfillment of the

Requirements for the

Degree of

MASTER OF SCIENCE

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October 1999

## PREFACE

This study was conducted to provide information regarding the kinetic interaction between midazolam and saquinavir. Midazolam is a frequently used medication and could have potentially catastrophic problems if there is an unrecognized interaction with saquinavir. This study explores this interaction *in vitro*.

## DEDICATION

To my family, Bridget, Lauren, and Natalie. Thank you for your support during this process and taking the time to hear me ramble on about what I am doing. I also want to extend my most grateful thanks to Svetlana Chershnikova Ph.D. for leading me through the process experiment and to Dr. Louis Cantilena for believing in me, that I would be able to undertake this study.

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# CHAPTER I: INTRODUCTION

## BACKGROUND OF THE PROBLEM

The rate of metabolism of midazolam is influenced by other drugs which can produce an increase or decrease in plasma concentrations of midazolam. The new antiviral drugs recently being prescribed for the treatment of acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus (HIV) may effect the rate of metabolism of midazolam because they use similar metabolic pathways. Any change in the rate of metabolism of midazolam can alter anesthetic requirements. A recent publication described the prolonged effects of midazolam with a patient who was taking protease inhibitor drug saquinavir (Merry, Mulcahy, Barry, Gibbons & Back, 1997). Less than one percent of midazolam is excreted in the urine (Dundee, Halliday, Harper & Brogden, 1984). This prolonged effect may be due to the competitive inhibition of the liver enzyme cytochrome P450 3A (CYP3A) by the protease inhibitors. Saquinavir is metabolized by the liver enzyme CYP3A4 (FDA, 1997). This in vitro study found that saquinavir decreased the rate of metabolism of midazolam.

Midazolam is a imidazobenzodiazepine derivative, chemically known as 8-Chloro-6-(Fluophenyl)-1-methyl-4-H-imidazol[1,5-a][1,4]benzodiazepine that is used as a short-acting sedative hypnotic (Backman, Aranko, Himberg & Olkkola, 1994). Midazolam is water soluble in a pH of 3.3, and becomes more lipid soluble at physiologic pH (Gerecke, 1983). The half-life of midazolam is 1.5 to 3 hours (Allonen et al., 1981).

Midazolam is the second most commonly used anesthetic in the United States

according to a 1996 survey conducted by the Council on Certification of Nurse Anesthetists Professional Practice Analysis (Council on Certification of Nurse Anesthetists, 1996) and the most commonly used preoperative anxiolytic for outpatient surgery (Twersky, 1995). Midazolam has a short half-life compared to diazepam. This property is preferred in anesthesia for short procedures and as a premedicant for general and regional anesthesia. Midazolam provides amnesia, which is especially effective for invasive procedures such as the insertion of pulmonary artery catheters and endoscopies. An inhibition of the metabolism of midazolam would cause unexpected sedation and respiratory depression (Merry et al., 1997). Patients prescribed protease inhibitors commonly require invasive procedures (Wastell, Corless, & Keeling, 1996). Midazolam is used for many of these procedures such as laporosopies, lumbar punctures, bronchoscopies, biopsies, and colonoscopies.

Clinical studies have revealed the problems of delayed metabolism and drug interaction (Backman, et al. 1994; Li, & Klotz, 1990; Olkkola et al., 1993). This variability of dosage and effect caused the Food and Drug Administration (FDA) to recommend that midazolam be given cautiously and in smaller doses to the elderly, the debilitated and those taking other drugs (FDA, 1987; FDA, 1988). Gascon and Dayer (1991) showed that the drugs erythromycin and ketoconazole inhibited the formation of midazolam metabolites as measured by the rate of formation the metabolites 1-hydroxymidazolam and the 4-hydroxymidazolam. These drugs are frequently used and have been implicated in adverse reactions when used in conjunction with midazolam.

Other drugs are likely to cause similar reactions when using the same enzyme pathway (Barry et al., 1997). Flockhart (1997) found that indinavir and saquinavir use the same enzyme as midazolam.

## RATIONAL AND SIGNIFICANCE OF THE PROBLEM

Treatment of acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus (HIV) includes the use of protease inhibitors for those patients who are symptomatic and asymptomatic (Department of Health and Human Services, 1997). In a city studied by Savioz, Lironi, Zurbuchen, Leissing, Kaiser & Morel (1995), they found the rate of AIDS infection to be as high as one percent of the population. The probability that midazolam will be given to these patients is high because AIDS patients frequently require surgical intervention to treat illnesses related to their disease. The incidence of surgical and diagnostic procedures is as high as 12 percent in these patients (Savioz et al., 1995).

A recent publication described the prolonged effects of midazolam in a patient who was taking protease inhibitor drugs (Merry, et al. 1997). The patient was sedated with midazolam 5 mg intravenously (IV) for a bronchoscopy procedure. The patient awoke spontaneously and was free of sedative effects when he was discharged home two hours later. During a second procedure in which 5 mg IV midazolam was given, Merry reported that after 5 mg of midazolam IV the patient remained sedated. Thirty minutes later the prolonged sedation was reversed with flumazenil 300 micrograms IV. Four hours prior to this second procedure the patient had taken 600mg of saquinavir. Protease

inhibitors are presently an integral part of the triple therapy now used in AIDS and HIV treatment (Department of Health and Human Services, 1997). The effect of these drugs on the rate of metabolism of midazolam needs to be further understood to assure the safety of these patients. Specifically, knowledge of the effects of saquinavir on the rate of metabolism of midazolam will allow for better clinical prediction.

## STATEMENT OF THE PROBLEM

The rate of metabolism of midazolam is unknown in patients who are taking the protease inhibitor saquinavir. Currently this drug is not recommended to be given concurrently with midazolam because of suspected inhibition (FDA, 1997). The effect on the rate of metabolism will help understand the consequences if they are used.

### Research Null Hypothesis

The null hypothesis for this study was:

There is no difference in the rate of metabolism for midazolam in the presence of saquinavir compared to the rate of metabolism of midazolam alone.

### Dependent Variable

The dependent variables which were measured in this study were:

1. The inhibition constant ($K_i$)for α-hydroxymidazolam.
2. The percent inhibition of α-hydroxymidazolam formation when metabolism takes place in the presence of saquinavir.

### Independent Variable

The independent variable for this study was the presence of saquinavir in the

concentrations of 0μM, 0.3 μM, 1.0μM, 3.0 μM and 10 μM.

## CONCEPTUAL FRAMEWORK

The conceptual framework for this study was based on the process of the biotransformation of drugs which has two phases. Phase I biotransformation adds a polar functional group on the parent compound. This is performed through oxidative, reductive, and hydrolytic reactions. These reactions generally result in a loss of pharmacologic activity. The phase II reactions are also called conjugation reactions. These reactions lead to a covalent bond between a functional group and one of the five compounds: acetate, amino acids, glucuronic acid, glutathione or sulfate. This forms a highly polar compound and is generally inactive and easily excreted in the urine. Although every tissue has the ability to metabolize drugs to some extent, the liver is the major organ for biotransformation (Benet, Kroetz, & Sheiner, 1996).

The mixed function oxidase system cytochrome P450 found in the microsomes of the hepatocyte is responsible for the biotransformation of drugs. The enzyme contains a hemeoprotein that combines with the drug to form a complex which is reduced by a proton transfer from NADPH. An oxygen molecule is then added to form an oxycytochrome P450. The next step is the transfer of a second proton from NADPH to form an activated oxygen species. In the final process, one atom of oxygen is released as water and the second oxygen is transferred to the substrate. Many different enzymes responsible for the process of biotransformation have been identified in the mixed function oxidase system. Sixty percent of drugs use the cytochrome 3A (CYP 3A)

isozyme found in the liver hepatocyte. CYP3A has the three isozymes 3A3, 3A4, and 3A5 (Wandel et al., 1994). An example of two drugs that use the same enzyme are erythromycin and midazolam (Hiller, Olkkola, Isohanni, & Saarnivaara, 1990). Competition for the same substrate-binding site is the most frequent mechanism for inhibition of drug metabolism (Barry et al., 1997). The protease saquinavir also uses the CYP3A isozyme for biotransformation which suggests an interaction between midazolam and saquinavir (Barry et al., 1997; Fitzsimmions, & Collins, 1997).

## DEFINITIONS OF TERMS

The following terms were used on this study: The use of the two brackets [term] means "the concentration of" the term that is placed in the brackets.

### α-Hydroxymidazolam

The metabolite formed by the hydroxylation of α or the one carbon in midazolam. This is the prominent metabolite in the biotransformation of midazolam (Thummel et al., 1994).

### 4-Hydroxymidazolam

The metabolite formed by the hydroxylation of the fourth carbon in midazolam (Thummel et al., 1994).

### Antiviral

An agent that destroys viruses or suppresses their replication (Friel, 1981).

### Bioavailability

The fraction of unchanged drug reaching the systemic circulation following administration by any route (Holford & Benet, 1998).

Biotransformation

The series of chemical alterations of a compound which occur within the body by enzymatic activity (Friel, 1981).

$C_{max}$

The maximum plasma concentration (Mahmood, Chamberlin, & Tammara, 1997)

Half-life (t 1/2)

The time, in a first-order chemical (or enzymatic) reaction, for half the substance (substrate) to be converted or to disappear. (Hensyl, 1990).

High Performance Liquid Chromatography (HPLC)

A process in which chemical substances are separated by differential movement through a stationary and mobile two-phase system. Material to be separated is injected into a column of a chosen absorbent. The substance least absorbed emerges first and the more absorbent substances emerge later (Willing & Tse, 1988).

In vitro

Referring to a process or reaction occurring in an artificial environment; as in a test tube or culture media. (Hensyl, 1990).

In vivo

Referring to a process within the living body (Hensyl, 1990).

$K_i$

The inhibition constant for Michaelis-Menten kinetics which describes the ability of a drug to inhibit the catalysis of a second drug (Fabre et. al., 1988).

$K_m$

The Michaelis constant that describes the affinity of an enzyme to a particular substrate. The $K_m$ is numerically equal to ½ the maximal velocity (Champe & Harvey, 1994).

Microsomes

Small spherical vesicles derived from the endoplasmic reticulum after disruption of cells and ultracentrifugation (Hensyl, 1990).

NADPH

A reduced form of Nicotinamide-adenine dinucleotide phosphate (NADP) the coenzyme of many oxidases (Hensyl, 1990).

Pharmacodynamics

The study of uptake, movement, binding, and interactions of pharmacologically active molecules at their tissue site(s) of action (Hensyl, 1990).

Pharmacokinetics

Movement of drugs within biological systems, as affected by uptake, distribution, binding, elimination, and biotransformation; particularly the rates of such movements (Hensyl, 1990).

Protease inhibitors

Drugs that inhibit the HIV protease enzyme that processes the viral proteins essential for the completion of the viral life cycle producing more infectious virons (Deeks, Smith, Holodniy & Kahn, 1997).

Therapeutic index

The [toxic]/[therapeutic] ratio which estimates the margin of safety of a therapy (Katzung, 1997).

V max

The maximal velocity of the biotransformation of a specific drug (Katzung, 1997).

Volume of Distribution (Vd)

The amount of drug present in the body in relation to the concentration of the drug in the plasma expressed in liters (Katzung, 1997).

## ASSUMPTIONS

The following assumptions were used for this study:

1. The aggragate of microsomes used in this study represent the human population. Microsomes from different human livers were pooled to obtain microsomes representative of the general population. The range of microsomal CYP3A4 content has been shown to vary by 10 to 100-fold from individual to individual (DeWaziers, Cunenc, Yang, Leroux, & Beaune, 1990; Guengrich & Turvey, 1991; Krohnbach, Mathys, Umeno, Gonzalez and Meyer1989; Watkins et al., 1985).
2. The concentrations of midazolam and the protease inhibitors were in the therapeutic

range. The theraputic range of midazolam is 300-1400 ng/ml for healthy young subjects with a body mass of 55 to 77 kg (Heizmann, Eckert and Zeigler, (1983). The concentration of midazolam used as a substrate in this in vitro experiment was 163 ng/ml- 3909 ng/ml which is in the range of 0.15 mg/kg or less of midazolam intravenously.

3. The assay developed by Hinkle (1997) was sensitive enough to detect the metabolites of midazolam produced.

## LIMITATIONS

The limitations of this study were as follows:

1. The pool of microsomes may not represent the general population. The isozyme make up of an individual may vary from person to person.
2. The samples of microsomes may have had exposure to other drugs, which may have influenced the activity of the P450 enzymes.

# CHAPTER II: REVIEW OF THE LITERATURE

## BACKGROUND

Midazolam is a short acting water-soluble benzodiazepine classified as an imidazobenzodiazepine derivative (Dundee et al., 1984). It is cardiorespiratory stable and useful in poor-risk, elderly and cardiac patients. The lack of long term active metabolites and the short half-life of midazolam makes it suitable as a long term sedative and as amnesic. The method of metabolism is by hydroxylation by the liver hepatocye to 1-hydroxymidazolam and 4-hydroxymidazolam with further conjugation and elimination in the urine.

The initial research of midazolam focused on the rates of clearance caused by differences in volume of distribution. Heizmann et al., (1983) explained the basic pharmacokinetic profile of midazolam and found that plasma concentrations of midazolam decreased to approximately 10 percent within two hours. The two metabolites of midazolam 1-hydroxymidazolam and 4-hydroxymidazolam were identified. The estimated volume of distribution (VD) and the bioavailability of midazolam ranged from 31 percent to 72 percent after oral administration. They attributed this wide range to the high liver extraction quota of midazolam (Heizmann et al., 1983).

## INTERACTIONS

Midazolam was approved in December of 1985 by the FDA and marketed in March of 1986 for use as a preoperative sedative, conscious sedative, and the induction of general

anesthesia. In February of 1987 a "Dear Doctor" letter indicates the need to have supportive measures available with the use of intravenous midazolam (FDA, 1987, p.5). Seventeen cases of respiratory and cardiac arrest associated with the use of intravenous midazolam were reported. The FDA stated that many of the cases involved the elderly and indicated a possible relationship between other drugs effecting the pharmacokinetics of midazolam. The FDA required a boxed warning be added to midazolam labeling. The need to reemphasize the close monitoring of patients during conscious sedation and attention to dosage recommendations prompted this action (FDA, 1988).

## METABOLISM OF MIDAZOLAM

The metabolism of midazolam involves the three isozymes CYP3A3, CYP3A4, and CYP3A5 of the P450 cytochrome mixed function oxidase system in the microsomes of the liver cell. The two isozymes CYP3A3 and CYP3A4metabolize midazolam to the same extent. The third isozyme CYP3A5 metabolizes midazolam 2.7 times faster than the isozymes CYP3A3 and CYP3A4. The isozyme CYP3A5 is found mainly in the kidney and to a lesser extent in the liver. The limiting factor of midazolam metabolism is the availability of these enzymes. Anything that affects the availability of the enzyme has a direct effect on the rate of metabolism of midazolam (Wandel et al., 1994).

## INHIBITION STUDIES OF MIDAZOLAM

Several drugs have been identified that induce or inhibit the metabolism of cyclosporin (Pichard et al., 1989). In this study the $K_I$ of midazolam in the presence of cyclosporin was found to be 40 μM. The $K_I$ identifies the ability of one drug to inhibit

the metabolism of another because of the affinity of the drug to the enzyme. Midazolam was among the drugs listed as inhibitory to the metabolism of cyclosporin. This study also suggests that other drugs could inhibit midazolam because they have a greater affinity for the same enzyme.

The inhibitory effects of drugs on midazolam was first published in 1990. In one case study an eight-year-old boy was given erythromycin after having received midazolam and the boy lost consciousness for 45 minutes. The authors concluded that from the enhanced effect of midazolam may have resulted from reduced hepatic clearence of midazolam caused by erythromycin (Hiller et al.,1990). Gascon and Dayer (1991) studied specific drugs that inhibited the formation of metabolites of midazolam by measuring the formation of the α-hydroxymidazolam and the 4-hydroxymidazolam. They confirmed that erythromycin inhibited the formation of midazolam metabolites.

In other studies the inhibitory effects of midazolam on other drugs was used as a probe by inhibiting known specific pathways. The affinity of the drug being studied for the enzyme can be determined with this probe. Midazolam is known to use the CYP3A3, CYP3A4, and CYP3A5 isozymes. The correlation of the amount CYP3A to the rate of midazolam allows midazolam to be used as a probe for the CYP3A system. This was shown in a study by Thummel, et al., (1994) identifying variability of CYP3A activity after liver transplantation.

## THE PHARMACOKINETICS OF MIDAZOLAM

Eagling, Back and Barry (1997) observed the pharmacokinetics of protease

inhibitors and their interaction with CYP3A mediated reactions. They noted if coadministered drugs are substrates for a common CYP pathway the elimination of one or both drugs may be impaired. The protease inhibitors are extensively metabolized by the CYP enzymes of the liver. These enzymes use the same enzyme pathway as midazolam. In a study by Fitzsimmons and Collins (1997), the protease inhibitors saquinavir and indinavir were shown to use the P450 CYP3A4 system for metabolism. They further demonstrated that the antifungal drug ketoconazole decreased the rate at which saquinavir metabolites were formed, thus slowing metabolism and elimination. Cato et al., (1997) revealed that the pharmacokinetics of ritonavir was altered by fluconazole, an antifungal drug. Kunze, Wienkers, Thummel and Trager (1996) showed fluconazole to be a moderately potent inhibitor of CYP3A. In the presence of fluconazole, there were statistically significant increases in maximum plasma concentrations of ritonavir (Cato et al. 1997).

There is little information available on the effects of midazolam when administered in combination with protease inhibitors. Although the FDA has issued warnings discouraging their combined use, it is possible that these two drugs may be used inadvertently together. In fact, one such case was recently reported in which an individual on the HIV protease inhibitor saquinavir was administered midazolam and did not wake spontaneously. This individual required a reversal agent, flumazenil, to recover and was not free of the sedative affects for five hours (Merry, Mulcahy, Barry, Gibbons & Back, 1997). These authors have indicated that if drugs have substrates of a common

P450 enzyme such as CYP3A and they are administered in combination, it is possible to presume that there would be a prolonged time of elimination for either one or both of those drugs. The specifics of exactly how these drugs interact during metabolism has not been investigated.

## SUMMARY

Previous studies suggest midazolam may be inhibited by protease inhibitors. There is only speculation regarding the drug interactions of midazolam and protease inhibitors. The purpose of this study is to quantify the effects of the protease inhibitor saquinavir on the metabolism of midazolam. The results will provide useful information to both the clinician, and the research scientist in the area of metabolism.

# CHAPTER III: METHODOLGY

## OVERVIEW

The experimental design of this study has been well established in pharmacological research. Human microsomes were prepared and pooled to provide a homogenous mixture of the P 450 isozymes found in the general population, thereby decreasing the effects of the explicit and unique characteristics found with each donor (Hinkel, 1997). Through carefully controlled conditions, the velocity of midazolam metabolite formation was measured without interfering factors. The protease inhibitor saquinavir was added to the midazolam to measure the change in velocity of metabolism.

## INVESTIGATIONAL REVIEW BOARD APPROVAL

This study had investigational review board approval under exempt status from the committee at the Uniformed Services University Of The Health Sciences. This study falls under the protocol T06156 for use of human subjects from entitled "The Effect of Saquinavir on the Rate of Metabolism of Midazolam."

## MICROSOME EXTRACTION

Microsomes were extracted from livers obtained through Washington Regional Transplant Consortium, Washington, D.C.. These livers were found unsuitable for transplantation but were deemed suitable for scientific research. Accompanying descriptive data pertaining to each liver sample were reviewed for possible interfering drug exposure. The livers had a code number associated to them for tracking the descriptive data with the sample. No other means of identification is possible.

Microsomes were extracted from the human liver samples following the protocol for extraction. The four microsome extracts were then pooled after determining similar activity, followed by measuring the protein concentration using the Bio-Rad Protien Assay, BioRad Laboratories (Richmond, CA) with bovine serum albumin as a standard. The microsomes were stored at $-80^0$ Celsius until used.

## ASSAY

To study the effect of midazolam on human microsomes, an accurate and sensitive measuring tool was required. The assay that Hinkel (1997) developed was used for this purpose. The same sample preparation, incubation conditions, and microsome concentrations were used.

## CHEMICALS AND REAGENTS

Midazolam, α-hydroxymidazolam was a gift from F. Hoffman-LaRoche, A. G. (Baser, Switzerland). The internal standard lorazepam was provided by Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile and water was purchased from Fisher Scientific (Pittsburgh, PA). Formic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). Constituents of the NADPH generating system were purchased from Sigma Chemical Co. (St. Louis, MO).

## STANDARD SOLUTIONS

Standard stock solutions of midazolam and its metabolite were prepared at a concentration of 0.32 mM in ethanol. Serial dilutions of the 0.32 mM standard were then used to make the appropriate working solutions of midazolam. A standard stock solution

of lorazepam was prepared at 3.11 mM in ethanol and further diluted to prepare the working solution at 5.0 μM. Stock and working solutions were stored at $5^0$ Celsius for the duration of the study which lasted one month.

## CALIBRATION CURVES

The calibration curve was prepared by using a ratio of midazolam to α-hydyoxymidazolam of 10:1. The concentrations of midazolam were 0.1, 0.5, 3.0, 6.0 and 12.0 μM. Two quality control points were added with midazolam concentrations of 1.0 and 10.0 μM. The pooled microsome suspension without the generating mixture was inoculated with each of the above-mentioned concentrations and 200 μL of Lorazepam (5 μM) then extracted per standard procedure, no incubation was done to these samples. There were five standard curve points and two quality control points added to the beginning of each HPLC run. Calibration curves were generated by least-square regression analysis of the analyte/internal standard peak-height ratio versus the concentration of the analyte.

## PILOT STUDY

After the components and tools for the study were assembled the experimental plan was tested. The 4-hydroxymidazolam metabolite was not used in this study because it has a very small signal peak and was not apparent at moderate to high levels of inhibition, whereas the α-hydroxymidazolam peak was better visualized due to its stronger signal. The pilot study included the incubation of midazolam with the protease

inhibitor in several concentrations below and including the C-max. This was used to elucidate problems with the signal detection prior to the full experiment.

## EXPERIMENT DESIGN

The experimental design consisted of four broad phases: Preparation of the microsomes, timed incubation with the midazolam and protease inhibitor, extraction of the metabolites from the remaining proteins, and HPLC analysis. The substrate concentrations were 0.5, 1.0, 3.0, 6.0 and 12.0 μM. Each of the five substrate concentrations were incubated with protease inhibitor concentrations of 0.0, 0.3, 1.0, 3.0 and 10.0 μM. Each substrate concentration was then run in quadruplet, to yield 100 data points plus controls and standards.

### Preparation of Microsomes

The microsomes stored in liquid nitrogen were thawed and 18.6 μL of microsomes were diluted with phosphate buffer to yield a final protein concentration of 0.2 mg protein/ml. Each microsomal suspension was then warmed in a shaking water bath at $37^0$ Celsius for three minutes after adding 10 uL of an NADPH generating system. The NADPH generating system was prepared by mixing 846 mg of glucose-6-phosphate, 252 mg of NADP+, 2460 μL of buffer, 540 μL of glucose-6-phosphate-dehydrogenase to total 3,000 μL which was enough solution for 300 samples.

### Incubation Phase

Ten μL of the five concentrations of MDZ were added at zero minutes and the

reaction incubated at $37^0$ C for 5 minutes at which time the test tubes were put into ice. Two hundred μL of Lorazepam (5 uM) was added to each sample and standards after the tubes were in ice.

Extraction Process

Five ml of acetonitrile was added to the incubation medium and the samples were vortexed for ten minutes. The test tubes were centrifuged at 2,000g and 5° C. for ten minutes. All incubation media were then transferred to clean tubes and labeled. The tubes were evaporated to dryness with a speed vacuum apparatus. Twenty μL of acetonitrile: water (1:1 v/v) was added and the tubes vortexed for three minutes. Two ml of acetonitrile was added to each tube and the tubes were again vortexed for three minutes and then centrifuged at 2,000g and 50 C. for ten minutes. The content of the test tubes were then transferred to clean test tubes and labeled. The test tubes were again evaporated to dryness with a speed vacuum apparatus. Following this, 20 μL of acetonitrile:water (1:1 v/v) was added to each test tube and vortexed for two minutes followed by the addition of 20 μL of water. The test tubes were vortexed for two minutes and then transferred to microvials and loaded onto the HPLC system.

HPLC Analysis

Separation of the midazolam, α-hydroxymidazolam, saqunavir and lorazepam was performed on a 150 x 2mm Prodigy 5μ, ODS (3), 100Å column by Phenomenex (Torrance, CA) using a linear gradient. Initial conditions were 15:85%, ACN (0.05% HCOOH): water. Ultra Violet detection was set at 220nm. Each of the samples took one hour to analyze on the HPLC system. The peaks of the midazolam, α-

hydroxymidazolam and lorazepam were visualized on the graph. If the peaks were not visualized at the known retention times, hand integration was performed to quantify the results. The data were then ready for entering the Statistical Package for the Social Sciences 8.0 (SPSS) (1997) spreadsheet database for analysis.

Once these calibration curves were established, the μM concentration of the experimental data set was calculated algebraically by using the peak heights of the experimental data for the Y variable. Grubbs' test for statistical outlyers was applied to the data to exclude extraneous points. The mean and standard deviation of the data was calculated and a 95% confidence interval was determined before any further analysis was conducted.

## DATA ANALYSIS

The data from the high performance liquid chromatography (HPLC) consisted of a graph of the retention time and the associated peak height. These peaks were measured through a process called integration. Were the base line of the graph is drawn to help exclude any baseline waver. The distance from the baseline to the top of the peak was then calculated. The peaks of interest were those of the internal standard and of the α-hydroxymidazolam. The retention times for α-hydroxymidazolam and the internal standard were 21.84 and 28.22 minutes respectively.

The α-hydroxymidazolam concentration was quantified by comparing ratios of α-hydroxymidazolam to internal standard peaks heights from the incubates with those produced with standard curves of authentic standards prepared in microsomal medium

with protein concentration 0.2 mg/ml. The standard curve is described by the formula: of α-hydroxymidazolam height / internal standard height =($T_g$) (C) + Yo. This is when $T_g$ is the slope of the standard curve, C is the concentration of calibrators, and Yo is the y intercept on the y-axis. The α-hydroxymidazolam concentration was then calculated by solving the above formula for C: C= (α-hydroxymidazolam/ internal standard – Yo)/ $T_g$. The result gives an intercept of -0.01115 and a slope of 1.0424.

The real concentrations of the α-hydroxy metabolite of midazolam were calculated were as well as the standard of deviation and coefficient of variation (See Tables 2, 3 and 4) using Microsoft Excel 97. The coefficient of variation was calculated for $p<.05$ and a $n=4$. The results are consistent for good data. The α-hydroxy metabolite of midazolam was produced in significantly smaller quantities when saquinavir was present when compared to the control. The anova significance was calculated using GraphPad Prism V2.01 (San Diego, CA) to be $p<.001$. (See Appendix C and Appendix D.)

Ki, and percentage of inhibition were determined for the 1' hydroxylation of MDZ by nonlinear regression analysis using the software package SPSS (1997). ANOVA factorial was used to test significance ($P<0.05$) of inhibition. The regression analysis measures the averages between points and generates a curve that follows the average path. The determination of Ki required graphing the 1/velocity versus 1/MDZ concentration to determine the slope of each line as is described by Lineweaver and Burke (1934). Following this a plot of the slopes versus the concentration of saquinavir

was done to determine the x-intercept. The x-intercept represents the Ki of saquinavir for midazolam.

# CHAPTER IV: RESULTS

## INTRODUCTION

Any change in the rate of metabolism of midazolam may alter its sedative effects. In this in vitro study, the effects of the protease inhibitor saquinavir on the metabolism of midazolam were measured using the microsomes of human livers.

## LIVER DONOR DEMOGRAPHICS

The human liver microsomes were obtained from four livers that were not used for transplantation. The drug history and other demographics were collected and reviewed for impact on this study (See Table 1).

**TABLE 1.**

**LIVER DONOR DATA**

| Liver Code Number | Significant Medication | Age | Sex |
|---|---|---|---|
| HLA 8 | Diltiazem X 15 years | 73 | Male |
| HLA 10 | None | 56 | Female |
| HLA 11 | Imipramine X 2.5 years | 16 | Female |
| HLA 20 | None reported | NA | NA |

NA= Not Available

Figure 1 and Appendix D shows the percent inhibition of the α-hydroxy

metabolite formation in the presence of saquinavir for each midazolam concentration. This decrease in metabolite was a monotonic decrease to the concentration of the protease inhibitor saquinavir that was in the solution. The increasing saquinavir concentration produces an increase in the percent inhibition with the 6 μM concentration of midazolam being aberrant. The mean percent inhibition for the α-hydroxymidazolam metabolite using therapeutic concentrations of the protease inhibitor and midazolam was 56.73% ±5.63

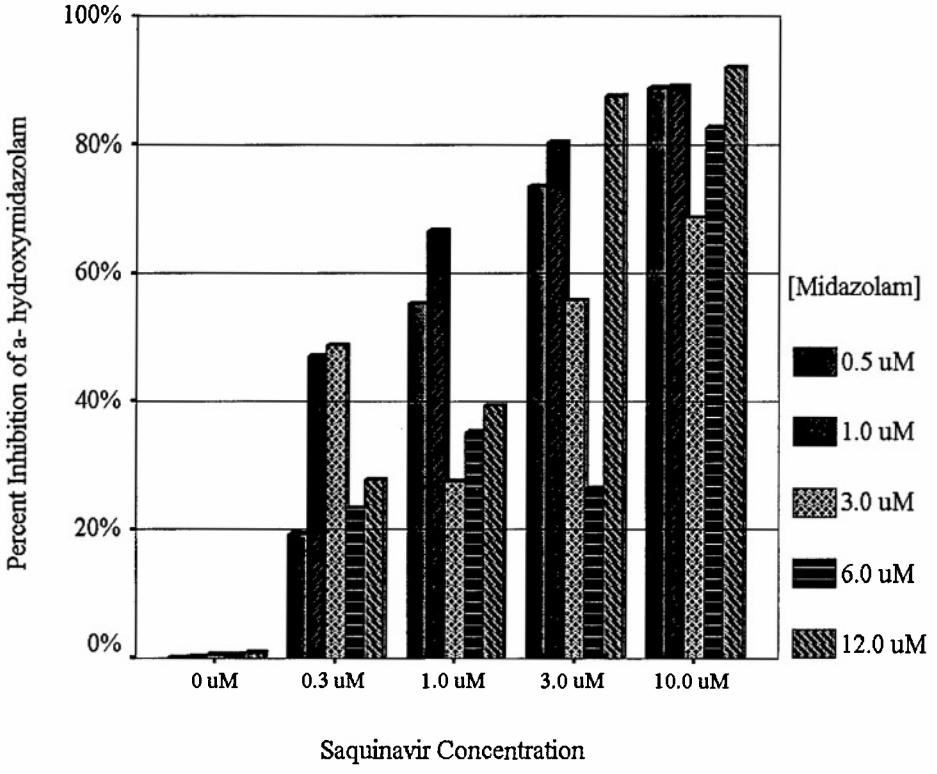
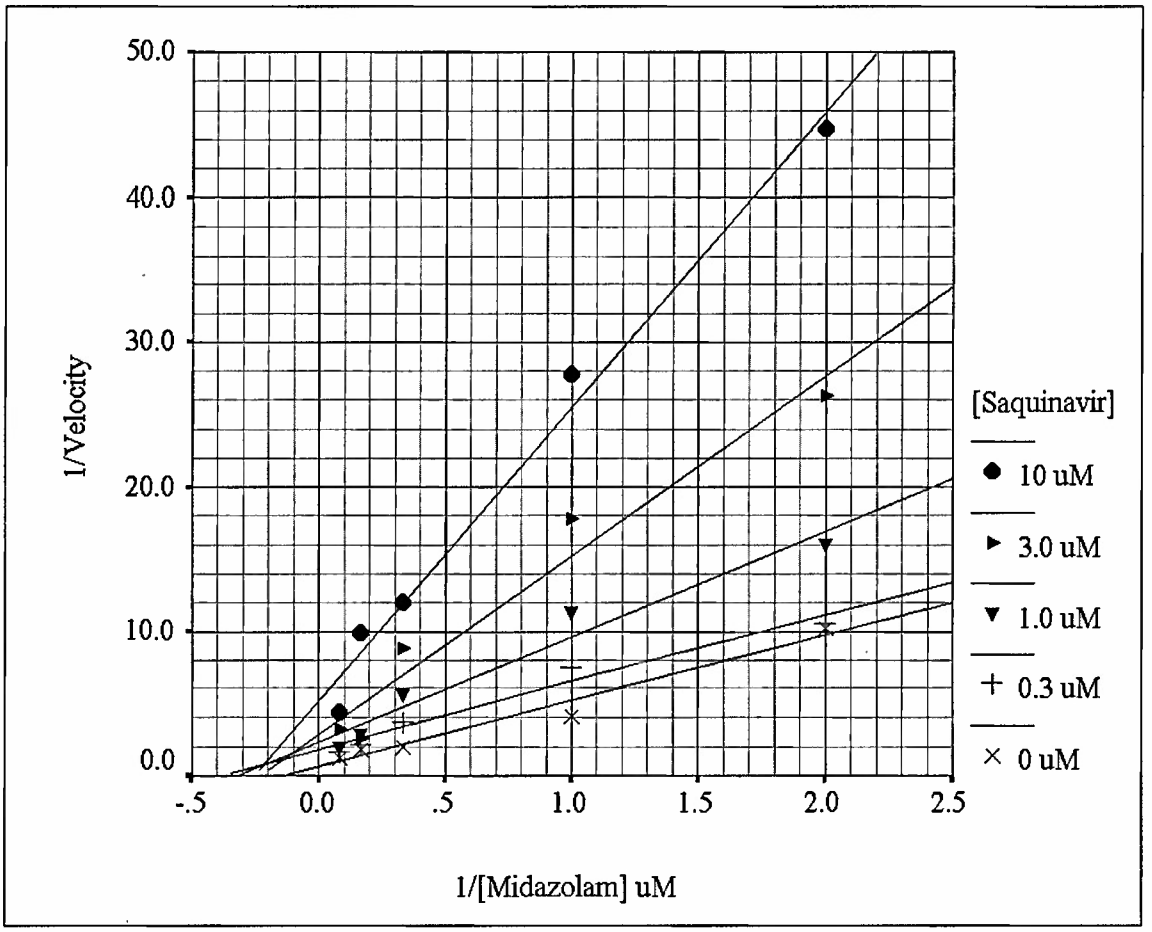



**FIGURE 1.**

**PERCENT INHIBITION OF α-HYDROXYMIDAZOLAM FORMATION**

The average of the four replicates real concentration of metabolite were then used as the velocity of metabolite formation for the Lineweaver-Burke plots (See Appendix E and Appendix F). The Lineweaver-Burke plot of the 1/ velocity of metabolite formation on the y-axis to 1/[substrate] on the x-axis produced a series of slopes for each concentration of the midazolam (See Figure 2 and Appendix G).

The slopes from the Lineweaver-Burke plots were then plotted on a graph of slope (on the y-axis) to [substrate] (on the x-axis) (See Appendix H).




PI Conc. = Protease inhibitor concentration SAQ = saquinavir Rsq = r squared
MDZ = midazolam Velocity = nmol/min/mg

**FIGURE 2.**

**LINEWEAVER BURKE PLOTS DEPICTING THE VELOCITY OF METABOLITE FORMATION FOR EACH SAQUINAVIR CONCENTRATION TO THE CONCENTRATION OF MIDAZLAM IN AN INVERSE RELATIONSHIP**

**TABLE 2.**

**DATA TABLE OF THE SLOPES OF THE LINEWAVER BURKE PLOTS FOR EACH CONCNETRATION OF SAQUINAVIR USED TO ESTIMATE KI.**

| Saq Conc. | Slopes | +/- error |
|---|---|---|
| 10 μM | 20.36 | 0.0022 |
| 3 μM | 12.4 | 0.0028 |
| 1 μM | 7.3 | 0.0036 |
| 0.3 μM | 4.66 | 0.0038 |
| 0 μM | 4.57 | 0.0007 |

Saq Conc. = saquinavir concentration

This plot gave the $K_i$ of saquinavir where the line of best fit intersects the x-axis (See Appendix H, Appendix I). The $K_i$ was determined to be at the 3.4μM concentration with the line of best fit having a $r^2 = 0.954$. The Km and Vmax of each protease inhibitor concentration was calculated by substitutions into the line equations of each Lineweaver-Burke plot (See Appendix J).

Figure 3 shows the Km for each concentration of saquinavir. The control is where no saquinavir was added to the reaction. These results showed that the Km is decreased by saquinavir during the metabolism of midazolam.

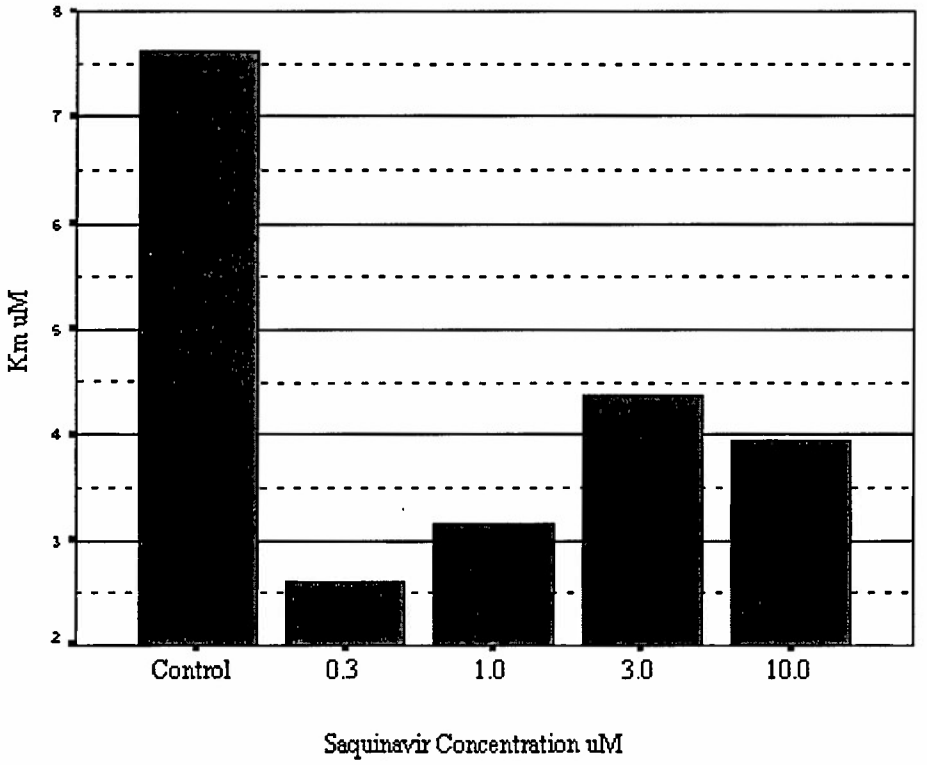



**FIGURE 3.**

**KM FOR EACH SAQUINAVIR CONCENTRATION**

Figure 4 shows the $V_{max}$ for each concentration of saquinavir. The control shows the $V_{max}$ with no saquinavir present during the metabolism of midazolam. There correlation between the concentration of the protease saquinavir and in the $V_{max}$ is $p=0.035$ ($\alpha=0.05$) with an $r^2=0.86$.

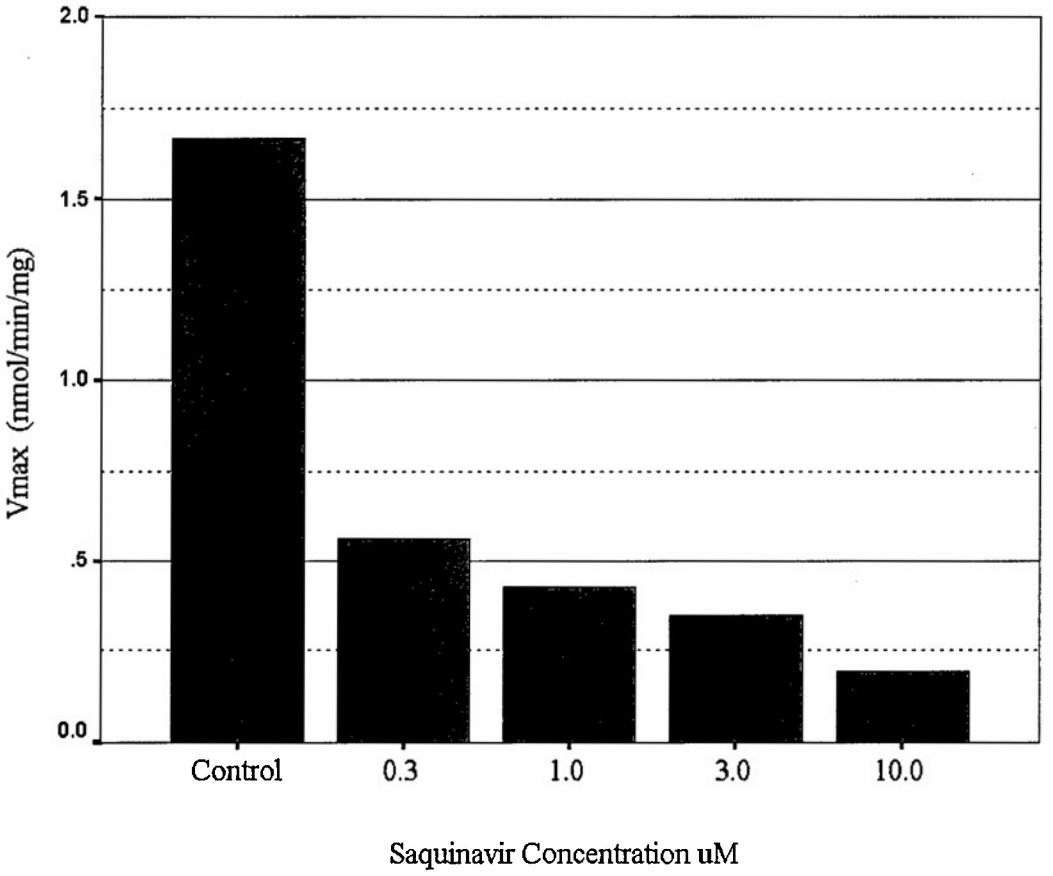



**FIGURE 4.**

**VMAX OF MIDAZOLAM METABOLISM IN THE PRESENCE OF SAQUINAVIR**

# CHAPTER V: DISCUSSION

## INTRODUCTION

Midazolam is a drug frequently used preoperatively and interoperatively, which can have its sedative effects prolonged by the interaction with other drugs. Past studies have shown inhibition between drugs using the same enzyme for metabolism (Eagling et al., 1997; Fitzsimmons and Collins, 1997; Iribarne et al, 1998). This study demonstrated that the protease inhibitor saquinavir inhibited the metabolism of midazolam in-vitro in therapeutic concentrations. The protease inhibitor saquinavir inhibited midazolam α-hydroxylation in a monotonic decrease to the concentration of saquinavir. This inhibition was characterized by a concentration related increase in $K_m$ values and decrease in $V_{max}$ values consistent with a mixed-type inhibition with a $K_i$ of 3.4 μM. This value strongly correlated to the documented $K_i$ values for saquinavir against CYP3A4 substrates. Others have found the $K_i$ with using methodone 15 μM, buprenorphine 7 μM (Iribarne et al, 1998), 3.0μM against testosterone (Eagling et al., 1997) and terfenadine metabolism ($K_i$ value of 0.7 μM) (Fitzsimmons and Collins, 1997)

## THEORETICAL IMPLICATIONS

The changing $K_m$ and $V_{max}$ appear to fit the properties for mixed inhibition as Iribarne et al., (1998) found with the interaction of saquinavir with methadone n-demethylation which is also a cyp3A4 metabolized drug. Further data is needed in the lower concentrations of saquinavir to confirm this type of inhibition. The effects of saquinavir on the metabolism of midazolam show that there is a common enzyme

metabolizing the two drugs. This is consistent with the findings of Eagling and others (1997) and those of Fitzsimmions and Collins (1997). The changing $K_m$ and $V_{max}$ data suggest that there are different sites of binding to the CYP 3A4 enzyme for the substrate and the inhibitor but further study is required to confirm this. There is the possibility that at the higher concentrations saquinavir the system was saturated although the data do not appear to show this.

## CLINICAL IMPLICATIONS

Saquinavir is packaged in two different formulations. The newest formulation called Fortovase, increases the $C_{max}$ value to approximately 1420 (CV 81%) and 2477 (CV 76%) ng/mL for healthy volunteers and HIV-infected patients, respectively (Roche, 1998). These $C_{max}$ ranges were selected for this study. This differs from the $C_{max}$ that was attained with the previous formulation named Invirase, which was in the range of 40 - 100 ng/ml as stated by Barry et al. (1997) and the product monograph (Roche, 1997). This may become more significant clinically as a potential mechanism of drug inhibition. The results of this study which were performed in this $C_{max}$ range show that inhibition of midazolam metabolism occurs if coadministered with saquinavir. These effects appear to have been observed in a case study (Merry, Mulcahy, et al., 1997).

The mixed inhibition suggests that there will be a prolonged inhibition of the metabolism of MDZ due to the binding of the CYP 3a enzyme to saquinavir. This binding is permanent and will exhibit prolonged inhibition after the $C_{max}$ of saquinavir

has dropped below the levels that were tested in this study.

## CONCLUSION

There was significant inhibition of midazolam metabolism in the presence of saquinavir when the concentrations were at $C_{max}$. The rate of midazolam metabolism was decreased *in-vitro* with a $K_i$ of 3.4 μM. Previously, saquinavir has been described to inhibit the human small intestinal microsomal P450 3A4-dependent terfenadine metabolism ($K_i$ value of 0.7 μM) (Fitzsimmons and Collins, 1997) and testosterone 6-hydroxylation with a $K_i$ of 3 μM (Eagling et al., 1997). Because of the low $K_i$ of saquinavir with midazolam, the possibility of an increase in oral dose absorption of midazolam from the gastrointestinal tract was expected. This was the case when ritonavir is used to increase the bioavailability of an oral dose of saquinavir. The first pass metabolism will be reduced and the blood levels of MDZ proportionally higher. Further research on the *in-vivo* metabolism of saquinavir and midazolam as well as further *in-vitro* studies using additional drug concentrations to further identify the type of inhibition needs to be done.

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## APPENDICIES

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# APPENDIX A: METABOLITE REAL CONCENTRATION CALCULATIONS AND STATISTICS

## APPENDIX A: METABOLITE REAL CONCENTRATION CALCULATIONS AND STATISTICS

| MDZ uM | Saq uM | M | IS | M/IS | concentration | average concentration | mean | st dev | CV |
|---|---|---|---|---|---|---|---|---|---|
| 0.5 | 0 | 7.97 | 73.48 | 0.10846 | 0.11474883 | 0.09761174 | 0.0906 | 0.01448 | 15.9803 |
| 0.5 | 0 | 6.96 | 72.45 | 0.09607 | 0.10285458 | | | | |
| 0.5 | 0 | 5.44 | 70.68 | 0.07697 | 0.08453193 | | | | |
| 0.5 | 0 | 6.14 | 75.89 | 0.08091 | 0.08831162 | | | | |
| 0.5 | 0.3 | 6.15 | 76.03 | 0.08089 | 0.08829487 | 0.0946443 | 0.08751 | 0.0128 | 14.6248 |
| 0.5 | 0.3 | 7.99 | 83.59 | 0.09559 | 0.10239348 | | | | |
| 0.5 | 0.3 | 5.37 | 73.59 | 0.07297 | 0.0806997 | | | | |
| 0.5 | 0.3 | 6.71 | 66.71 | 0.10058 | 0.10718914 | | | | |
| 0.5 | 1 | 3.02 | 69.66 | 0.04335 | 0.05228617 | 0.06236223 | 0.04852 | 0.00915 | 18.8645 |
| 0.5 | 1 | 5.09 | 86.14 | 0.05909 | 0.06738242 | | | | |
| 0.5 | 1 | 5.1 | 73 | 0.06986 | 0.07771732 | | | | |
| 0.5 | 1 | 5.14 | 119.2 | 0.04312 | 0.05206301 | | | | |
| 0.5 | 3 | 1.59 | 67.47 | 0.02357 | 0.03330374 | 0.03803544 | 0.0285 | 0.00702 | 24.6352 |
| 0.5 | 3 | 2.5 | 115.99 | 0.02155 | 0.03137317 | | | | |
| 0.5 | 3 | 2.78 | 77.26 | 0.03598 | 0.045215 | | | | |
| 0.5 | 3 | 3.35 | 101.85 | 0.03289 | 0.04224985 | | | | |
| 0.5 | 10 | 0.74 | 89.7 | 0.00825 | 0.01861052 | 0.02235178 | 0.01215 | 0.0027 | 22.2 |
| 0.5 | 10 | 1.13 | 85.24 | 0.01326 | 0.0234138 | | | | |
| 0.5 | 10 | 0.91 | 71.75 | 0.01268 | 0.02286338 | | | | |
| 0.5 | 10 | 1.25 | 86.75 | 0.01441 | 0.02451945 | | | | |
| 1 | 0 | 16.12 | 72.22 | 0.22321 | 0.22482298 | 0.2419021 | 0.24101 | 0.0265 | 10.9937 |
| 1 | 0 | 29.51 | 105.55 | 0.27958 | 0.2789058 | | | | |
| 1 | 0 | 19.8 | 88.36 | 0.22408 | 0.22566375 | | | | |
| 1 | 0 | 18.02 | 75.98 | 0.23717 | 0.23821585 | | | | |
| 1 | 0.3 | 10.07 | 75.91 | 0.13266 | 0.13795687 | 0.13315662 | 0.12765 | 0.0082 | 6.42358 |
| 1 | 0.3 | 14.34 | 121.33 | 0.11819 | 0.12407837 | | | | |
| 1 | 0.3 | 12.05 | 91.21 | 0.13211 | 0.13743463 | | | | |
| 1 | 0.3 | | | #DIV/0! | #DIV/0! | | | | |
| 1 | 1 | 7.1 | 74.7 | 0.09505 | 0.10187665 | 0.08794555 | 0.08052 | 0.01142 | 14.1783 |
| 1 | 1 | 6.53 | 84.79 | 0.07701 | 0.0845772 | | | | |
| 1 | 1 | 5.69 | 84.05 | 0.0677 | 0.07564019 | | | | |
| 1 | 1 | 6.78 | 82.34 | 0.08234 | 0.08968818 | | | | |
| 1 | 3 | 3.92 | 108.5 | 0.03613 | 0.04535567 | 0.05622591 | 0.04746 | 0.00978 | 20.6166 |
| 1 | 3 | 4.04 | 78.09 | 0.05174 | 0.06032694 | | | | |
| 1 | 3 | 3.6 | 82.98 | 0.04338 | 0.05231545 | | | | |
| 1 | 3 | 4.78 | 81.58 | 0.05859 | 0.06690558 | | | | |
| 1 | 10 | 2.28 | 74.37 | 0.03066 | 0.04010675 | 0.03600087 | 0.02638 | 0.0046 | 17.4461 |
| 1 | 10 | 1.56 | 73.09 | 0.02134 | 0.03117168 | St dev = Standard Deviation | | | |
| 1 | 10 | 2.01 | 67.26 | 0.02988 | 0.03936472 | M= metabolite IS= Internal Standard | | | |
| 1 | 10 | 1.89 | 80 | 0.02363 | 0.03336032 | CV= Coefficient of Variation | | | |

## APPENDIX A: METABOLITE REAL CONCENTRATION CALCULATIONS AND STATISTICS CONTIUED.

| MDZ | SAQ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| uM | uM | 1-OH | IS | M/IS | Concentration | Average concentration. | mean | st dev | CV |
| 3 | 0 | 31.91 | 67.94 | 0.46968 | 0.46126849 | 0.51120578 | 0.52173 | 0.09454 | 18.1212 |
| 3 | 0 | 32.01 | 69.06 | 0.46351 | 0.45535031 | | | | |
| 3 | 0 | 32.3 | 65.74 | 0.49133 | 0.48203808 | | | | |
| 3 | 0 | 47.29 | 71.39 | 0.66242 | 0.64616625 | | | | |
| 3 | 0.3 | 18.92 | 77.51 | 0.2441 | 0.24486379 | 0.26716024 | 0.26734 | 0.02517 | 9.41448 |
| 3 | 0.3 | 21.99 | 77.35 | 0.28429 | 0.28342327 | | | | |
| 3 | 0.3 | 23.09 | 78.69 | 0.29343 | 0.29218928 | | | | |
| 3 | 0.3 | 19.86 | 80.23 | 0.24754 | 0.24816461 | | | | |
| 3 | 1 | 12.35 | 73.13 | 0.16888 | 0.17270364 | 0.1784504 | 0.17487 | 0.00416 | 2.37673 |
| 3 | 1 | 13.12 | 74.7 | 0.17564 | 0.17918723 | | | | |
| 3 | 1 | 13.63 | 76.4 | 0.1784 | 0.18184192 | | | | |
| 3 | 1 | 14.79 | 83.77 | 0.17655 | 0.18006882 | | | | |
| 3 | 3 | 9.29 | 76.36 | 0.12166 | 0.12740768 | 0.11305102 | 0.1067 | 0.01318 | 12.3494 |
| 3 | 3 | 8.64 | 83.57 | 0.10339 | 0.10987692 | | | | |
| 3 | 3 | 8.86 | 79.6 | 0.11131 | 0.11747487 | | | | |
| 3 | 3 | 7.16 | 79.18 | 0.09043 | 0.09744462 | | | | |
| 3 | 10 | 7.05 | 81.83 | 0.08615 | 0.09334578 | 0.0831915 | 0.07557 | 0.01062 | 14.0529 |
| 3 | 10 | 7.24 | 88.48 | 0.08183 | 0.08919402 | | | | |
| 3 | 10 | 6.1 | 84.83 | 0.07191 | 0.07967961 | | | | |
| 3 | 10 | 5.58 | 89.44 | 0.06239 | 0.07054658 | | | | |
| 6 | 0 | 47.34 | 75.4 | 0.62785 | 0.6130062 | 0.54127571 | 0.55308 | 0.05071 | 9.1691 |
| 6 | 0 | 40.67 | 78.26 | 0.51968 | 0.50923335 | | | | |
| 6 | 0 | 40.52 | 77.38 | 0.52365 | 0.5130433 | | | | |
| 6 | 0 | 42.62 | 78.76 | 0.54114 | 0.52981998 | | | | |
| 6 | 0.3 | 36.74 | 88.42 | 0.41552 | 0.40930959 | 0.46551449 | 0.4153 | 0.02133 | 5.13689 |
| 6 | 0.3 | 45.25 | 69.56 | 0.65052 | 0.6347502 | | | | |
| 6 | 0.3 | 32.77 | 75.07 | 0.43653 | 0.42946397 | | | | |
| 6 | 0.3 | 42.34 | 107.5 | 0.39386 | 0.38853421 | | | | |
| 6 | 1 | 35.59 | 91.08 | 0.39076 | 0.38555544 | 0.34770851 | 0.3513 | 0.0264 | 7.51383 |
| 6 | 1 | 29.07 | 86.34 | 0.33669 | 0.33369156 | | | | |
| 6 | 1 | 29.5 | 87.68 | 0.33645 | 0.33345996 | | | | |
| 6 | 1 | 25.37 | 74.33 | 0.34132 | 0.33812707 | | | | |
| 6 | 3 | 42.71 | 121.29 | 0.35213 | 0.34850259 | 0.39262242 | 0.39812 | 0.05168 | 12.9817 |
| 6 | 3 | 47.61 | 101.66 | 0.46833 | 0.4599702 | | | | |
| 6 | 3 | 44.78 | 110.73 | 0.40441 | 0.39865181 | | | | |
| 6 | 3 | 35.2 | 95.75 | 0.36762 | 0.36336509 | | | | |
| 6 | 10 | 7.66 | 74.31 | 0.10308 | 0.10958462 | 0.10112801 | 0.09427 | 0.0087 | 9.23047 |
| 6 | 10 | 7.83 | 80.54 | 0.09722 | 0.10396022 | St dev = Standard Deviation | | | |
| 6 | 10 | 6.76 | 71.64 | 0.09436 | 0.1012184 | M= metabolite | | IS= Internal Standard | |
| 6 | 10 | 5.62 | 68.2 | 0.0824 | 0.08974879 | CV= Coefficient of Variation | | | |

## APPENDIX A: METABOLITE REAL CONCENTRATION CALCULATIONS AND STATISTICS CONTINUED.

| MDZ uM | Saq uM | M | IS | M/IS | concentration | average concentration | Mean | st dev | CV |
|---|---|---|---|---|---|---|---|---|---|
| 12 | 0 | 58.98 | 71.69 | 0.82271 | 0.79993661 | 0.83095187 | 0.85504 | 0.11066 | 12.9417 |
| 12 | 0 | 71.72 | 70.52 | 1.01702 | 0.98633954 | | | | |
| 12 | 0 | 53.69 | 66.03 | 0.81312 | 0.79073326 | | | | |
| 12 | 0 | 54.28 | 70.74 | 0.76732 | 0.74679807 | | | | |
| 12 | 0.3 | 42.29 | 72.08 | 0.58671 | 0.57353766 | 0.6037039 | 0.61815 | 0.02862 | 4.62973 |
| 12 | 0.3 | 53.43 | 81.79 | 0.65326 | 0.6373795 | | | | |
| 12 | 0.3 | 46.62 | 74.35 | 0.62703 | 0.61222228 | | | | |
| 12 | 0.3 | 50.46 | 83.32 | 0.60562 | 0.59167616 | | | | |
| 12 | 1 | 40.5 | 77.19 | 0.52468 | 0.51403125 | 0.50898597 | 0.51942 | 0.01981 | 3.81463 |
| 12 | 1 | 33.2 | 64.98 | 0.51093 | 0.50083781 | | | | |
| 12 | 1 | 41.31 | 75.91 | 0.5442 | 0.53275496 | | | | |
| 12 | 1 | 39.88 | 80.1 | 0.49788 | 0.48831986 | | | | |
| 12 | 3 | 20.73 | 70.48 | 0.29413 | 0.29285704 | 0.31206679 | 0.31415 | 0.0191 | 6.08002 |
| 12 | 3 | 20.87 | 67.77 | 0.30795 | 0.3061219 | | | | |
| 12 | 3 | 29.02 | 92.19 | 0.31478 | 0.31267531 | | | | |
| 12 | 3 | 24.58 | 72.35 | 0.33974 | 0.33661291 | | | | |
| 12 | 10 | 16.51 | 235.64 | 0.07006 | 0.07791061 | 0.07647052 | 0.06856 | 0.00259 | 3.77209 |
| 12 | 10 | 17.04 | 262.4 | 0.06494 | 0.07299364 | St dev = Standard Deviation | | | |
| 12 | 10 | 21.13 | 298.76 | 0.07073 | 0.07854488 | M= metabolite IS= Internal Standard | | | |
| 12 | 10 | 19.97 | 291.43 | 0.06852 | 0.07643294 | CV= Coefficient of Variation | | | |

## APPENDIX B: DATA FOR METABOLITE/ INTERNAL STANDARD ANOVA TABLE

APPENDIX B: DATA FOR METABOLITE/ INTERNAL STANDARD ANOVA TABLE

| Saq 0μM | Saq 0.3μM | Saq 1.0μM | Saq 3.0μM | Saq 10.0μM |
|---|---|---|---|---|
| 0.10846 | 0.08089 | 0.04335 | 0.02357 | 0.00825 |
| 0.09607 | 0.09559 | 0.05909 | 0.02155 | 0.01326 |
| 0.07697 | 0.07297 | 0.06986 | 0.03598 | 0.01268 |
| 0.08091 | 0.10058 | 0.04312 | 0.03289 | 0.01441 |
| 0.22321 | 0.13266 | 0.09505 | 0.03613 | 0.03066 |
| 0.27958 | 0.11819 | 0.07701 | 0.05174 | 0.02134 |
| 0.22408 | 0.13211 | 0.0677 | 0.04338 | 0.02988 |
| 0.23717 | | 0.08234 | 0.05859 | 0.02363 |
| 0.46968 | 0.2441 | 0.16888 | 0.12166 | 0.08615 |
| 0.46351 | 0.28429 | 0.17564 | 0.10339 | 0.08183 |
| 0.49133 | 0.29343 | 0.1784 | 0.11131 | 0.07191 |
| 0.66242 | 0.24754 | 0.17655 | 0.09043 | 0.06239 |
| 0.62785 | 0.41552 | 0.39076 | 0.35213 | 0.10308 |
| 0.51968 | 0.65052 | 0.33669 | 0.46833 | 0.09722 |
| 0.52365 | 0.43653 | 0.33645 | 0.40441 | 0.09436 |
| 0.54114 | 0.39386 | 0.34132 | 0.36762 | 0.0824 |
| 0.82271 | 0.58671 | 0.52468 | 0.29413 | 0.07006 |
| 1.01702 | 0.65326 | 0.51093 | 0.30795 | 0.06494 |
| 0.81312 | 0.62703 | 0.5442 | 0.31478 | 0.07073 |
| 0.76732 | 0.60562 | 0.49788 | 0.33974 | 0.06852 |

Saq = Saquinavir

## APPENDIX C: ANOVA RESULTS OF METABOLITE/INTERNAL STANDARD COMPARING CONTROL TO INCUBATES WITH SAQUINAVIR.

## APPENDIX C: ANOVA RESULTS OF METABOLITE/INTERNAL STANDARD COMPARING CONTROL TO INCUBATES WITH SAQUINAVIR.

| Parameter | | | | |
|---|---|---|---|---|
| Table Analyzed | Inverse MDZ & [Saq] | | | |
| **TWO-WAY ANOVA** | | | | |
| Source of Variation | % of total variation | P value | | |
| Column Factor | 31.94 | P<0.0001 | | |
| Row Factor | 49.29 | P<0.0001 | | |
| Source of Variation | P value summary | Significant? | | |
| Column Factor | *** | Yes | | |
| Row Factor | *** | Yes | | |
| Source of Variation | Df | Sum-of-squares | Mean square | F |
| Column Factor | 4 | 3292 | 823.1 | 491700 |
| Row Factor | 4 | 5081 | 1270 | 758800 |
| Residual | 75 | 0.1255 | 0.001674 | |
| Number of missing values | -25 | | | |

Df= degrees of freedom
[Saq]= concentration of Saquinavir

## APPENDIX D: PERCENT INHIBITION OF α-HYDROXYMIDAZOLAM FORMATION FOR EACH MIDAZOLAM CONCENTRATION AND SAQUINAVIR CONCNETRATION

## APPENDIX D: PERCENT INHIBITION OF α-HYDROXYMIDAZOLAM FORMATION FOR EACH MIDAZOLAM CONCENTRATION AND SAQUINAVIR CONCNETRATION

| MDZ Conc. | SAQ 0 μM | SAQ 0.3 μM | SAQ 1.0 μM | SAQ 3.0 μM | SAQ 10μM |
|---|---|---|---|---|---|
| 0.00 μM | 0.1085 | 0.2410 | 0.5217 | 0.5413 | 0.8550 |
| 0.30 μM | 19.3215 | 47.0341 | 48.7594 | 23.2737 | 27.7045 |
| 1.00 μM | 55.2654 | 66.5886 | 27.4438 | 35.0971 | 39.2519 |
| 3.00 μM | 73.7257 | 80.3078 | 55.7301 | 26.4475 | 87.5006 |
| 10.00 μM | 88.7986 | 89.0554 | 68.6448 | 82.5844 | 91.9813 |

MDZ Conc. = midazolam concentration SAQ = saquinavir

# APPENDIX E: MEAN OF THE REAL CONCENTRATION OF SAQUINAVIR USED TO DO CALCULATIONS FOR LINEWAVER-BURKE PLOTS

## APPENDIX E: MEAN OF THE REAL CONCENTRATION OF SAQUINAVIR USED TO DO CALCULATIONS FOR LINEWAVER-BURKE PLOTS

| SAQ 0.0uM | SAQ 0.3uM | SAQ 1.0uM | SAQ 3.0uM | SAQ 10.0uM |
|---|---|---|---|---|
| 97611.740 | 94644.30 | 62362.23 | 38035.44 | 22351.78 |
| 241902.10 | 133156.62 | 87945.55 | 56225.91 | 36000.87 |
| 511205.78 | 267160.24 | 178450.40 | 113051.02 | 83191.50 |
| 541275.71 | 465514.49 | 347708.51 | 392622.42 | 101128.01 |
| 830951.87 | 603703.90 | 508985.97 | 312066.79 | 229411.56 |

SAQ = saquinavir

## APPENDIX F: INVERSE REAL CONCENTRATIONS OF THE METABOLITES LISTED BY INVERSE MIDAZOLAM CONCENTRATION USED TO CREATE LINEWEAVER BURKE PLOTS

## APPENDIX F: INVERSE REAL CONCENTRATIONS OF THE METABOLITES LISTED BY INVERSE MIDAZOLAM CONCENTRATION USED TO CREATE LINEWEAVER BURKE PLOTS

| 1/[MDZ] | Saq 0μM | | | Saq 0.3μM | | |
|---|---|---|---|---|---|---|
| X | Y | SD | N | Y | SD | N |
| 2 | 10.24467 | 0.013889 | 4 | 10.56588 | 0.012277 | 4 |
| 1 | 4.133904 | 0.025418 | 4 | 7.509953 | 0.007866 | 4 |
| 0.3333333 | 1.956159 | 0.090699 | 4 | 3.743072 | 0.024145 | 4 |
| 0.1666667 | 1.847487 | 0.048649 | 4 | 2.148161 | 0.114055 | 4 |
| 0.08333334 | 1.203439 | 0.106155 | 4 | 1.656441 | 0.027455 | 4 |

| 1/[MDZ] | Saq 1μM | | | Saq 3μM | | |
|---|---|---|---|---|---|---|
| X | Y | SD | N | Y | SD | N |
| 2 | 16.03535 | 0.012498 | 4 | 26.29127 | 0.006735 | 4 |
| 1 | 11.37067 | 0.010953 | 4 | 17.78539 | 0.009387 | 4 |
| 0.3333333 | 5.603798 | 0.003987 | 4 | 8.845564 | 0.01264 | 4 |
| 0.1666667 | 2.875972 | 0.025323 | 4 | 2.546976 | 0.04958 | 4 |
| 0.08333334 | 1.964691 | 0.019008 | 4 | 3.204442 | 0.018323 | 4 |

| 1/[MDZ] | Saq 10μM | | |
|---|---|---|---|
| X | Y | SD | N |
| 2 | 44.73917 | 0.002587 | 4 |
| 1 | 27.77711 | 0.004415 | 4 |
| 0.3333333 | 12.02046 | 0.010188 | 4 |
| 0.1666667 | 9.888457 | 0.008347 | 4 |
| 0.08333334 | 4.358978 | 0.002481 | 4 |

X= x-axis data
Y= y-axis data
N= number of data points
SD= standard deviation

# APPENDIX G: LINEWEAVER BURKE PLOT RESULTS WITH $R^2$, SLOPE, Y INTERCEPT PRESENTED

## APPENDIX G: LINEWEAVER BURKE PLOT RESULTS WITH $R^2$, SLOPE, Y INTERCEPT PRESENTED

| | Saq 0 | Saq 0.3 | Saq 1 | Saq 3 | Saq 10 |
|---|---|---|---|---|---|
| Variables | | | | | |
| Slope | 4.574 ± 0.4607 | 4.664 ± 0.5119 | 7.303 ± 0.8760 | 12.40 ± 1.518 | 20.36 ± 1.366 |
| Y-intercept | 0.5992 ± 0.4674 | 1.782 ± 0.5193 | 2.336 ± 0.8887 | 2.850 ± 1.540 | 5.167 ± 1.385 |
| X-intercept | -0.131 | -0.3822 | -0.3199 | -0.2299 | -0.2538 |
| 1/slope | 0.2186 | 0.2144 | 0.1369 | 0.08066 | 0.04912 |
| 95% Confidence Intervals | | | | | |
| Slope | 3.108 to 6.040 | 3.035 to 6.293 | 4.516 to 10.09 | 7.568 to 17.23 | 16.01 to 24.70 |
| Y-intercept | -0.8881 to 2.086 | 0.1298 to 3.435 | -0.4916 to 5.164 | -2.050 to 7.750 | 0.7586 to 9.575 |
| Goodness of Fit | | | | | |
| $r^2$ | 0.9705 | 0.9651 | 0.9586 | 0.957 | 0.9867 |
| Sy.x | 0.7397 | 0.8219 | 1.406 | 2.437 | 2.193 |
| Is slope significantly non-zero? | | | | | |
| F | 98.55 | 82.99 | 69.51 | 66.71 | 222.2 |
| DFn, DFd | 1.000, 3.000 | 1.000, 3.000 | 1.000, 3.000 | 1.000, 3.000 | 1.000, 3.000 |
| P value | 0.0022 | 0.0028 | 0.0036 | 0.0038 | 0.0007 |
| Deviation from zero? | Significant | Significant | Significant | Significant | Significant |
| Data | | | | | |
| Number of X values | 5 | 5 | 5 | 5 | 5 |
| Max. number of Y replicates | 1 | 1 | 1 | 1 | 1 |
| Total number of values | 5 | 5 | 5 | 5 | 5 |

## APPENDIX H: PLOT OF SLOPES TO SAQUINAVIR CONCENTRATION RESULTING IN A X-INTERCEPT WHICH IS THE KI.

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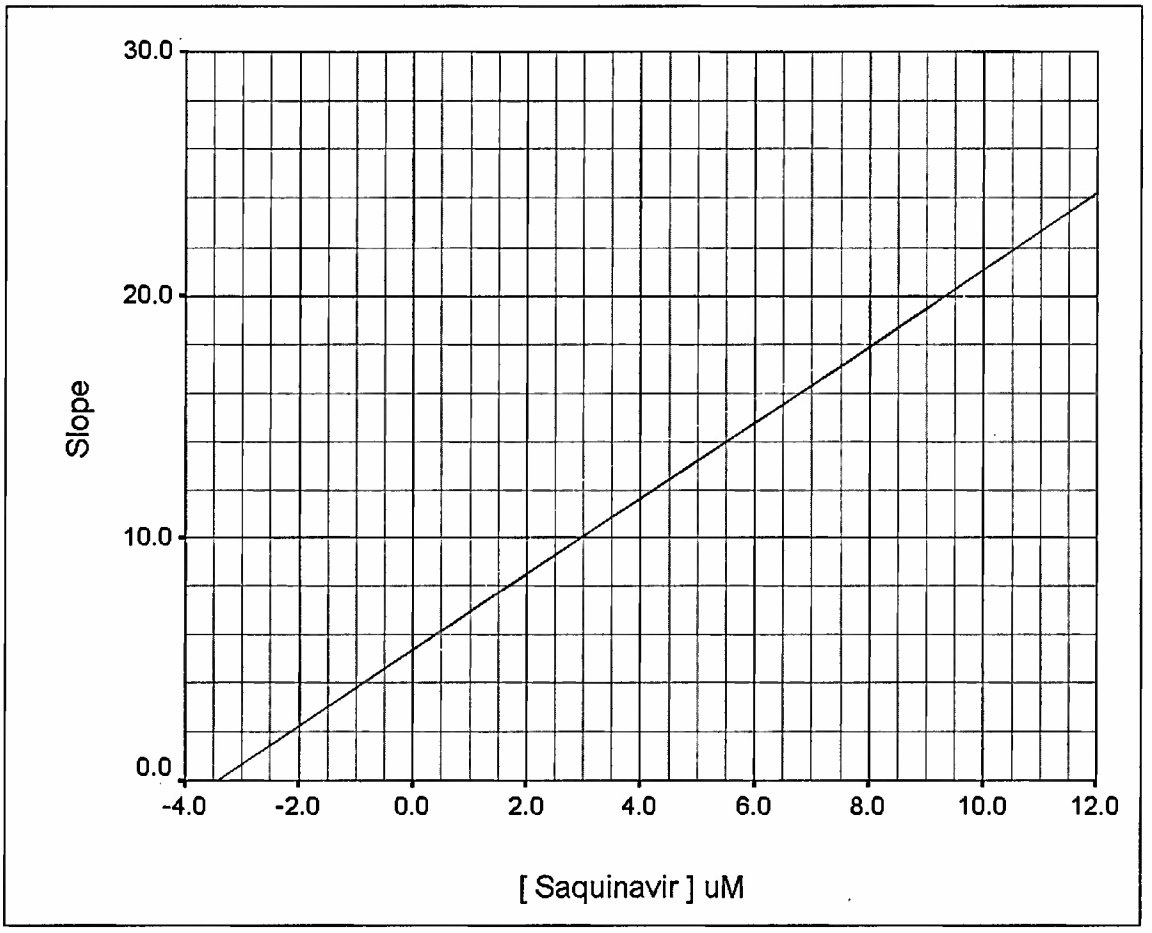

# APPENDIX I: KI PLOT TABULAR DATA, SHOWING THE $R^2$ AND X INTERCEPT (KI)

## APPENDIX I: KI PLOT TABULAR DATA, SHOWING THE $R^2$ AND X INTERCEPT (KI)

| Variables | Slopes |
|---|---|
| Slope | 1.569 ± 0.1969 |
| Y-intercept | 5.372 ± 0.9241 |
| X-intercept | -3.425 |
| 1/slope | 0.6375 |
| 95% Confidence Intervals | |
| Slope | 0.9419 to 2.195 |
| Y-intercept | 2.432 to 8.313 |
| Goodness of Fit | |
| $r^2$ | 0.9548 |
| Sy.x | 1.638 |
| Is slope significantly non-zero? | |
| F | 63.43 |
| DFn, DFd | 1.000, 3.000 |
| P value | 0.0041 |
| Deviation from zero? | Significant |
| Data | |
| Number of X values | 5 |
| Maximum number of Y replicates | 1 |
| Total number of values | 5 |
| Number of missing values | 0 |

## APPENDIX J: VMAX AND KM FOR EACH PROTEASE INHIBITOR CONCENTRATION

## APPENDIX J: VMAX AND KM FOR EACH PROTEASE INHIBITOR CONCENTRATION

| PI Conc. (μM) | Vmax (nmol/min/mg) | Km (μM) |
|---|---|---|
| 0 μM | 1.667 | 7.617 |
| 0.3 μM | .562 | 2.618 |
| 1.0 μM | .427 | 3.154 |
| 3.0 μM | .351 | 4.379 |
| 10.0 μM | .193 | 3.938 |

PI Conc. = protease inhibitor concentration

## APPENDIX K: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PRINT OUT DEPICTING THE INTEGRATED PEAK HEIGHTS

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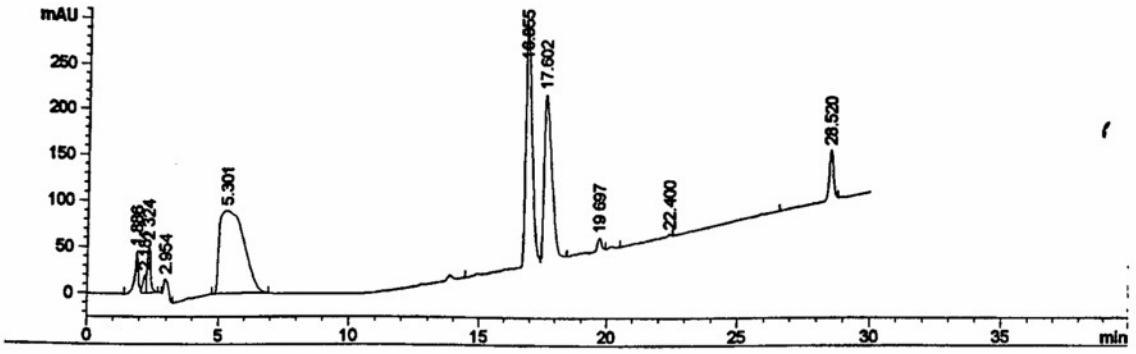


Height Percent Report with Performance

# APPENDIX L: IN VITRO MIDAZOLAM METABOLISM SOP

## APPENDIX L: IN VITRO MIDAZOLAM METABOLISM SOP

The pooled microsomes were analyzed using the BioRad protein assay giving a concentration 10.73 mg/ml. This required 18.6 µL to be used to attain a concentration of 0.2 mg/ml after dilution

### MATERIALS:

1. Midazolam, α-hydroxymidazolam
2. Lorazepam
3. HPLC grade acetonitrile
4. Formic acid
5. NADPH generating system: NADP+, G6P, G6PDH
6. human liver microsome samples that are pooled to a concentration of 10.73 mg/ml.
7. Protein assay: Bio-Rad Standard 11 dye, Bio-Rad, stock protein solutions ranging from 0.5-0.05 mg/ml, microliter plates.
8. 50 mM potassium phosphate buffer (pH 7.4)
9. Ethanol

### METHOD:

1.Prepare stock solution of MDZ (3.2 mM):

   a. Formula weight MDZ = 325.8

   b. Make 5 ml of stock solution

APPENDIX L (CONTINUED): IN VITRO MIDAZOLAM METABOLISM SOP

c. Weigh 5.213 mg MDZ in a volumetric flask

d. Add up to 5 ml ethanol

e. Store in refrigerator

2. Prepare stock solution of α -Hydroxymidazolam (3.2 mM):

a. Formula weight = 341.8

b. Make 50 ml of stock solution

c. Weigh 5.469 mg α - Hydroxymidazolam in a volumetric flask

d. Add up to 50 ml ethanol

e. Store in refrigerator

3. Prepare 3.11 mM stock solution of Lorazepam (internal standard)

4. Make MDZ dilutions from stock (10ml each) to use in incubations:

a. 800uM------- 2500 uL stock, 7500 uL distilled water

b. 600uM-------- 1900 uL stock, 8100uL distilled water

c. 400uM-------- 1250 uL stock, 8750 uL distilled water

d. 200uM-------- 625 uL stock, 9375 uL distilled water

e. 100uM-------- 313 uL stock, 9687 uL distilled water

f. 50uM--------- 156 uL stock, 9844 uL distilled water

5. Make a mix of MDZ and it's metabolites to use to construct calibration curves:

a. 1000 uL MDZ stock (3.2 mM)

APPENDIX L (CONTINUED): IN VITRO MIDAZOLAM METABOLISM SOP

b. 1000 uL I -Hydroxymidazolarn (3.2 mM)

c. 1000 uL 4-Hydroxymidazolam (3.2 mM)

d. 1000 uL distilled water

e. The resulting solution is 800 uM

6. Take 4ml of the MDZ mix (800 uM) and put 1 ml and 1 ml into two separate tubes. Add 1 ml water to one tube and add 3 ml water to the other tube. The result is 400 uM and 200 uM concentrations of the MDZ mix. Continue to make serial dilutions of the mix according to the following:

4ml - 800uM solution

1 ml + 1 ml H20 = 400 uM (2ml)

1ml + 3ml H20 = 200 uM (4ml) Take 2 ml from this to make next dilution.

1ml + Iml H20 = 100 uM (2ml)

1ml + 3-rnl H20 = 50 uM (4ml) Take 2 ml for next dilution.

1ml + I ml H20 = 25 uM (2ml)

1ml + 4ml H20 = 10 uM (5 ml) Take 2 ml for next dilution.

1ml + 1ml H20 = 5 uM (2 ml)

1ml + 4ml H20 = 1 uM (5ml) Take 2 ml for next dilution.

1mi + 1ml H20 = 0.5uM (2ml)

1ml + 4ml H20 = 0. 1 uM (5ml) Take 2 ml for next dilution.

1ml + 1ml H20 = 0.05uM (2ml)

APPENDIX L (CONTINUED): IN VITRO MIDAZOLAM METABOLISM SOP

1ml + 4ml H20 = 0.01 uM (5ml) Take 2 ml for next dilution.

1ml + 1ml H20 = 0.005 uM (2ml)

1ml + 4ml H20 = 0.001 uM (5ml)

7. The pooled microsome samples will be subjected to 5 different concentrations of MDZ and all work will be done in quadruplicate.
8. Perform protein assay on all microsome samples. (See SOP for protein assay)
9. Prepare NADPH generating system:
   a. Mix: 650 ug G6P, 252 mg NADP+, 2490 uL buffer, 540 uL G6PDH, (556 u/ml)
   b. This will total 30uL, enough for 300 samples.
10. Add sufficient microsome suspension to each test tube to obtain a final protein concentration of 0.2 mu/ml after dilution. Base this on the calculated protein content of each microsome sample. Add 10 uL of NADPH generating system and up to 0.980 ul, of 50 mM potassium phosphate buffer.
11. Place test tubes in a shaking water bath for 3 min. at 37 C. Tubes used for standards should be kept on ice
12. Add 10 uL of MDZ solution at 0 min. (The tubes used for standards will have added, then kept on ice without incubation)
13. Incubate tubes 5 min.
14. Plunge tubes into ice at 5 min. (see chart for time schedule.)

APPENDIX L (CONTINUED): IN VITRO MIDAZOLAM METABOLISM SOP

15. Add 200 uL of the internal standard stock solution to each test tube
16. Add 5 ml acetonitrile to each tube of incubation
17. Vortex each tube for 10 min.
18. Centrifuge tubes at 2000 g; 5 C; 10 min.
19. Transfer to clean tubes and label.
20. Evaporate tubes to dryness with speed vac.
21. Add 20 uL acetonitrile:water (1: 1 V/V).
22. Vortex each tube for 3 min.
23. Add 2 ml of acetonitrile to each tube.
24. Centrifuge tubes at 2000 g; 5 C; 10 min.
25. Transfer to clean tubes and label.
26. Evaporate tubes to dryness with speed vac.
27. Add 20 uL acetonitrile:water (1: 1 v/v) to each tube.
28. Vortex for 2 min. at speed 5.
29. Add 20-uL water to each tube.
30. Vortex 2 min. at speed 5
31. Transfer each sample from test tube to microvial. Label and load onto HPLC system.

CHROMATOGRAPHY:

1. Use of a Hewlett Packard 1050 HPLC system will be employed.

APPENDIX L (CONTINUED): IN VITRO MIDAZOLAM METABOLISM SOP

1. System fitted with micro bore tubing and Prodigy 5 u ODS (3) 1 00 A column.
2. Flow rate = 0.2 ml/min.
3. Run time = 60 min.
4. Temperature = 35 C.
5. Injection vol.=10uL
6. Lamp wavelength = 220 nm.
7. Solvent A = H20
8. Solvent B = .05% Formic acid in acetonitrile. (pH 4. 1)
9. Flow rate gradient:

a. Time in min.: flow rate ) 0:0.2, 2:0.2, 3.5:0.25, 5:0.25, 30:0.25, 32:0.25, 33:0.4, 39.8:0.4, 40:0.25, 45:0.25, 55:0.2.

11. Gradient conditions:

a. Time in min.: percent formic acid in acetonitrile ) 0: 15, 2:15, 3.5:15, 5:15, 30:45, 32:98, 33:98, 39.8:98, 40:98, 45:15, 55:15.